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Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment

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Aquaculture Extension Manual No. 37

Laboratory Manual of Standardized Methods for Antimicrobial Sensitivity Tests for Bacteria Isolated from Aquatic Animals and Environment

Lila Ruangpan and Eleonor A. Tendencia



**Southeast Asian Fisheries Development Center
Aquaculture Department**

Government of Japan Trust Fund

December 2004

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Laboratory Manual of Standardized Methods for Antimicrobial Sensitivity Tests for Bacteria Isolated from Aquaculture Animals and Environment

December 2004

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FOREWORD

Worldwide trade in aquaculture products demands strict adherence to standards for good quality and safe seafood for the consumers. Thus, information on good aquaculture practices, specifically those that have direct influence on marketable products, should be disseminated. One of the most needed methods concerns health maintenance and disease control. While fish products are generally considered wholesome and healthy, farming practices during production may affect their quality. The use and misuse of antibiotics in aquaculture have led to rejection of exported products. Thus, farmers and all sectors involved in the production and the marketing process need to know about the hazards of employing practices that may lead to product contamination.

Realizing the importance of this issue and in response to growing need to evaluate the extent of antibiotic usage in aquaculture, the Government of Japan has, since the year 2000, funded the Southeast Asian Fisheries Development Center (SEAFDEC) through the Trust Fund for the Regional Fish Disease Project entitled “Development of Fish Disease Inspection Methodologies for Artificially-Bred Seeds.” Under this regional project, various research studies were conducted, and two authors (Dr. Lila Ruangpan, Chanthaburi Coastal Fisheries Research and Development Center, Thailand and Ms. Eleonor A. Tendencia, SEAFDEC Aquaculture Department, Philippines) of this manual were among the study leaders. The manual is one of the important outputs of a collection of studies related to antibiotic usage in order to come up with guidelines for its prudent usage. It offers a complete guide for testing bacterial susceptibility and resistance through the use of simple techniques for disk-agar diffusion tests, and a guide to do a more thorough study to test therapeutic levels using microbial inhibitory concentration.

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December 2004

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The Government of Japan Trust Fund through SEAFDEC Aquaculture Department provided financial support for the research project and publication of this manual. We would like to express our sincere thanks to Dr. Yasuo Inui and Dr. Kazuya Nagasawa, the former and current Fish Disease Experts and Leaders of the Regional Fish Disease Project, for their encouragement and critical suggestions; and the staff of the Fish Health Section, SEAFDEC Aquaculture Department, for their kind cooperation, especially to Dr. Celia R. Lavilla-Pitogo for carefully reading the manuscript and Dr. Edgar C. Amar for serving as the over-all coordinator in the preparation of drafts. Thanks also to Dr. Hisatsugu Wakabayashi, Emeritus Professor of Fish Pathology Laboratory at the University of Tokyo, for reviewing the manuscript.

INTRODUCTION

Bacteria are ubiquitous in the environment and may cause disease and mortality in cultured shrimp and fish both in the hatchery and grow-out ponds. Accordingly, antimicrobial agents are widely used by farmers especially in the intensive culture system. Misuse of drugs and non-compliance of treatment regimens among users can cause treatment to be less effective and prolong the duration of disease. Furthermore, they may increase the prevalence of transferable resistance genes to the bacteria in the environment and in food fish that may indirectly affect human health due to limited therapeutic options.

Since several antimicrobial agents are widely used in various areas of shrimp and fish culture, testing for the susceptibility and resistance of bacteria to different agents need to be conducted. The data gathered from the tests are essential in the selection of the most effective antimicrobial agents against pathogenic bacteria. Furthermore, the values obtained in the tests are useful in determining the dosage regimen of each antimicrobial.

Different methods have been employed in laboratories to determine the susceptibility of microorganisms to antimicrobial agents. Two main methods for the *in vitro* determination of the susceptibility of microorganisms against antimicrobials are the disk agar diffusion test, wherein antibiotic-impregnated disks are used with an agar medium, and the dilution techniques, wherein the test microorganism is exposed to increasing concentration of an antibiotic either in broth or agar.

The disk agar diffusion method is the most widely used laboratory technique for antimicrobial susceptibility test. This method is simple, requires a short duration, and easy to perform. It also provides an accurate determination of the susceptibility of an organism to a measured amount of test antibiotics. However, only a few antimicrobials have veterinary-specific interpretative criteria; therefore, human interpretative criteria are used for the majority of the chemotherapeutants. Human interpretative criteria could, however, vary from veterinary-specific data due to strain differences in the bacterial species.

The dilution technique, using broth and agar media, is used to measure quantitatively the *in vitro* activity of antimicrobial agents in terms of susceptibility and resistance by bacteria (Thornsberry *et al.*, 1977; Thornsberry and McDougal, 1983). The minimal inhibitory concentration (MIC) using agar dilution is one of the appropriate *in vitro* tests widely used among several laboratories (McDonald *et al.*, 1995). The results obtained by MIC dilution test can give an indication as to the concentration of antimicrobial agent needed to inhibit or eradicate the infectious organism. Basically, a series of plates is prepared with an agar medium to which various concentrations of the antimicrobial agents are added. The plates are then

inoculated with a suitable standardized suspension of the test bacteria. After the plates are incubated overnight under a certain temperature, examination of the test results is performed, MIC values are read, and drug resistance of the bacterial strains is determined.

In both techniques, it is important that control strains be included to check the quality of batches of media and to monitor the performance of the assay protocol. The selection of the control strain should be based on the kind of bacteria being investigated in any set of experiments. The National Committee for Clinical Laboratory Standards (NCCLS) recommended the use of the following organisms for the quality control of antimicrobial susceptibility test: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Actinobacillus pleuropneumoniae*, *Haemophilus somnus*, *Campylobacter jejuni*, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae*.

The series of procedures, materials preparation, as well as the interpretative criteria to obtain the final results in antimicrobial sensitivity testing are influenced greatly by the method used, which must be standardized and carefully controlled. The standard methods found in this manual are written for the students, teachers, laboratory technicians, researchers, and scientists who work in this field.

REFERENCES

- McDonald, C.L., W.E. Maher and R.J. Fass. 1995. Revised interpretation of oxacillin MICs for *Staphylococcus epidermidis* based on MecA detection. Antimicrob. Agents Chemother., 39: 982-984.
- Thornsberry, C., T.L. Gaven and E.H. Gerlach. 1977. New developments in antimicrobial agent susceptibility testing. Cumitech, 6: 1-13.
- Thornsberry, C. and L.K. McDougal. 1983. Successful use of broth microdilution in susceptibility tests for methicillin-resistant (heteroresistant) staphylococci. Clin. Microbiol., 18: 1084-1091.

CHAPTER I

Bacterial Isolation, Identification, and Storage

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PRINCIPLE

Bacterial isolation, purification and identification are the first steps to bacteriological studies. Isolation is done to obtain pure bacterial cultures. Bacteria are usually isolated from fish kidney and spleen; and from the hepatopancreas, lymphoid organ and muscles of shrimp. These tissues are monitor organs that usually harbor the disease-causing bacteria during infection.

To obtain a pure bacterial culture is the first step to bacterial identification. Pure culture is essential in the study of the morphology, physiology, biochemical characteristics, and susceptibility to antimicrobial agents of a particular bacterial strain.

Pure cultures are best obtained by using solid media, by streak plate or pour plate method. Streak plate, if properly done, is the most practical method. In the streak plate method, a loopful of the inoculum is placed near the periphery of the plate with agar medium and spread or streaked on the upper portion of the plate with parallel overlapping strokes. The inoculum is streaked over other portions of the plate so that isolated colonies could be observed in the last streaked area.

The identification of a bacterial pathogen is important in fish diagnosis. Treatment could be implemented only after the causative agent or the bacterium has been identified. Bacterial species differ in morphological, physiological and biochemical characteristics and those can be used when coding or labelling them (Appendix 1.1). Therefore, identification is accomplished by performing several morphological, physiological and biochemical tests. Results of these tests are compared to established taxa or identification schemes (Appendix 1.2).

Bacterial cultures should be preserved for future study. Storing in appropriate medium preserves bacterial cultures. The simplest method is by sub-culturing or by transferring the organism to fresh solid medium that has a minimal nutrient content to prevent bacterial overgrowth. The bacteria are allowed to grow before storing in the refrigerator or are covered with paraffin oil and stored at room temperature in the dark. Another simple method is by deep-freezing of the bacterial culture, stocked in a broth medium with glycerol. Glycerol is added to prevent the dessication of bacterial cells. Bacterial cultures may also be preserved by freeze-drying or lyophilization. In this method, water is removed from the frozen bacterial suspension by sublimation under vacuum.

Bacterial cultures should be properly labeled or coded before storage. It is important to label the tube or vial for storing bacterial cultures with an indelible ink. The label or code should include the reference number and other pertinent information such as source of sample (host animal, location), date of isolation, special properties, identification, name of the person who isolated the organism and the date of preparation of the stock culture.

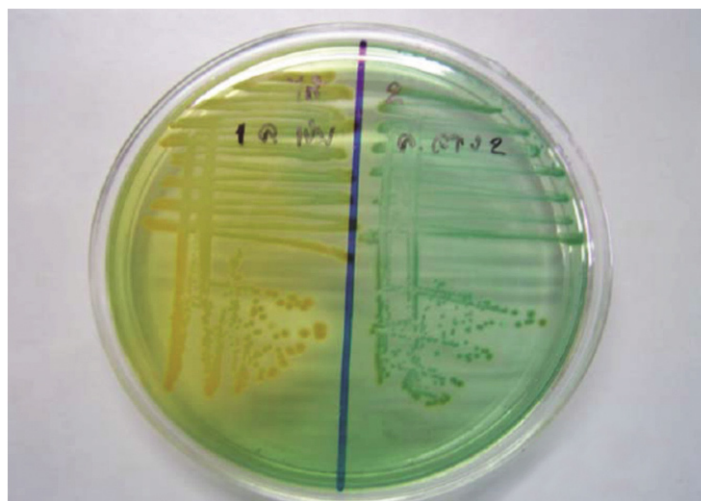
ISOLATION

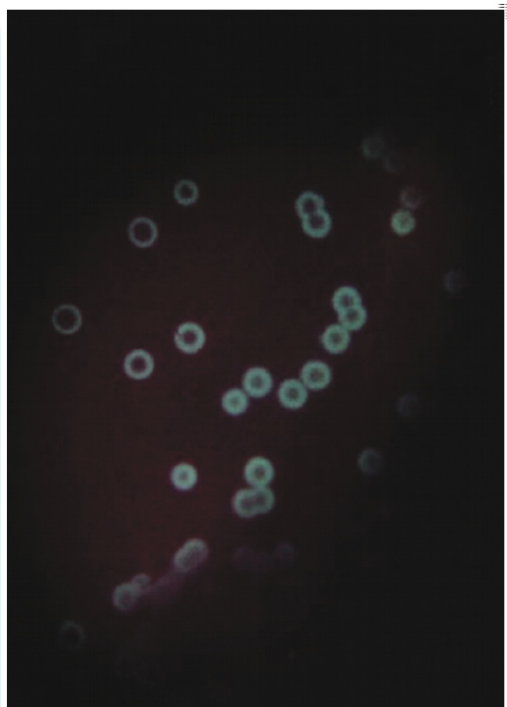
Media

Bacterial isolation can be done using a general medium, wherein various bacteria can grow, and selective media that allows growth of specific genera. Examples of general media are nutrient agar (NA), tryptic soy agar (TSA), and brain heart infusion agar (BHIA). Examples of selective media are thiosulfate citrate bile sucrose agar (TCBS) for vibrios, and glutamate starch phenol red agar (GSP) for aeromonads and pseudomonads. Media are supplemented with 1-2% sodium chloride (NaCl) if to be used for marine species. Adjust the pH of the culture media to 7.2-7.4 by adding 0.1 N NaOH.

Streaking

- 1 Using inoculating loop, get samples of shrimp (such as the hepatopancreas and muscle) and fish (kidney, spleen) tissues and streak onto the upper one-fourth portion of an agar plate with parallel overlapping strokes. Use one agar plate for each animal sample. The plate can be divided into half and streaked with two different tissues from the same sample. Be sure to label the plate.
- 2 Flame the loop and allow it to cool. Turn the plate at right angle. Overlap the previous streak once or twice and repeat the streaking process on one-half of the remaining area.
- 3 Repeat procedure 2.
- 4 Incubate plates overnight at 30°C. Photo at right shows a streaked plate after incubation.



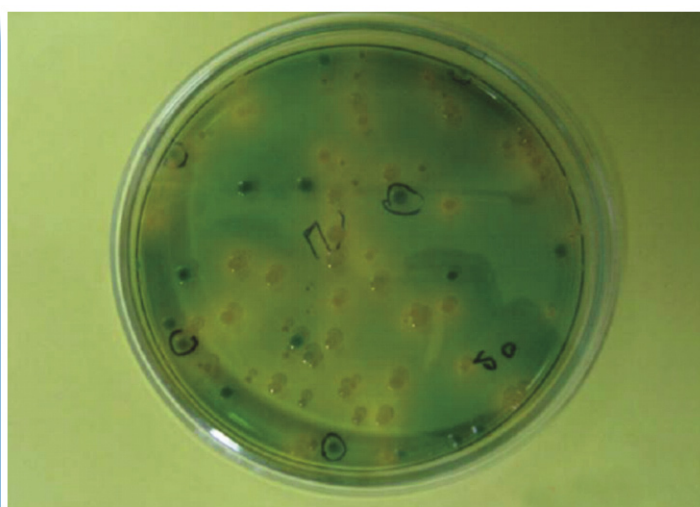


5

After incubation for 16-20 hours, check for bacterial growth. Check for luminescence under dark conditions, marking the luminous colonies on the plate with a pentel pen. Isolated colonies should be observed in the last streaked area.

6

Select representative bacterial colonies based on the difference in shape, size and color. Mark selected colonies from each plate. Subculture onto trypticase soy agar (TSA) plate and incubate overnight.



STORAGE

- 1 Observe the colonies on the agar plate to determine the purity of the culture. Pure cultures should show the same colony characteristics and not overlapping.



- 2 Select a pure well-isolated colony. Stab each strain into 2 tubes of 1.2% TSA, label and incubate. These will serve as stock cultures.

- 3 Keep the stock cultures in the lowest compartment of the refrigerator (8-12°C) or at room temperature until use. Do not stock cultures in these conditions for over 6 months.

4

Purified bacterial cultures may also be stocked in nutrient broth with 20% glycerol and stored at -80°C until use. Bacterial cultures may be stocked in this condition for 2 years.

NOTE: *The stocks may be coded or labeled based on the source of the samples, date of collection, color of the colony and ability of the colony to emit light (Appendix 1.1).*

IDENTIFICATION

Bacterial isolates may be identified using conventional methods based on their morphological, biochemical and physiological characteristics. The following are important biochemical tests for the identification of bacterial genera that are important in aquaculture:

1. Gram reaction
2. Oxidase test
3. Motility
4. Oxidation and fermentation test
5. O/129 sensitivity test
6. Sensitivity to novobiocin

The scheme for the identification of bacterial genera that are important in aquaculture is presented in Appendix 1.2.

Further biochemical characterization must be carried out if there is a need to identify up to the species level.

NOTE: *Aseptic techniques must be observed all throughout.*

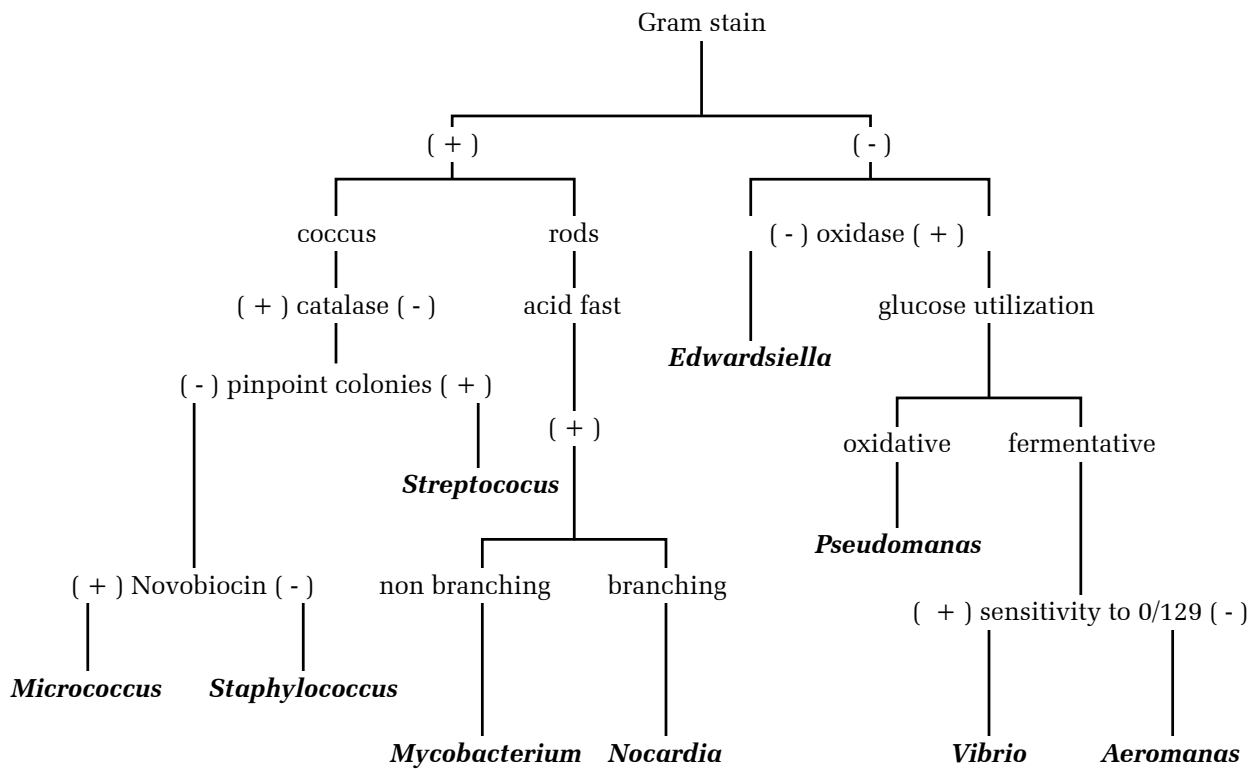
REFERENCES

- Bailey, W.R. and E.G. Scott. 1966. Diagnostic Microbiology, Second Edition. Toppan Company Ltd., Japan, 342 pp.
- Tonguthai, K., S. Chinabut, T. Somsiri, P. Chanratchakol and S. Kanchanakhan. 1999. Diagnostic Procedures for Finfish Diseases. Aquatic Animal Health Research Institute, Bangkok, Thailand.

APPENDIX 1.1. Example of important bacterial characteristics for *Vibrio* species, that are taken into consideration when coding or labeling isolated bacteria.

Source of sample	Host animals and geographical location are important informations to use as code of drug resistant bacteria.
Color of the colony on TCBS	<i>Vibrio</i> bacteria normally appear as green or yellow colonies on TCBS medium. Many vibrios that form green colonies are more virulent to shrimp.
Special characteristics	Luminous bacteria emit light in the dark. They are, therefore, easy to collect for special or future studies. Luminous bacteria may be labeled as LB.

APPENDIX 1.2. Identification scheme for the identification of bacterial genera that are important in aquaculture (modified from Tonguthai *et al.*, 1999).



CHAPTER 2

Disk Diffusion Method

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PRINCIPLE

This method is based on the principle that antibiotic-impregnated disk, placed on agar previously inoculated with the test bacterium, pick-up moisture and the antibiotic diffuse radially outward through the agar medium producing an antibiotic concentration gradient. The concentration of the antibiotic at the edge of the disk is high and gradually diminishes as the distance from the disk increases to a point where it is no longer inhibitory for the organism, which then grows freely. A clear zone or ring is formed around an antibiotic disk after incubation if the agent inhibits bacterial growth.

MEDIA

The disk diffusion method is performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility, low in sulfonamide, trimethoprim, and tetracycline inhibitors, and gives satisfactory growth of most bacterial pathogens.

The inoculum for the disk diffusion method is prepared using a suitable broth such as tryptic soy broth. This medium is prepared according to manufacturer's instructions, dispensed in tubes at 4-5 ml and sterilized. Sterile 0.9% salt solution may also be used.

Media are supplemented with 1-2% sodium chloride (NaCl) if intended for marine organisms.

Preparation of agar medium

1

Prepare MHA from the dehydrated medium according to the manufacturer's instructions. Media should be prepared using distilled water or deionized water.

2

Heat with frequent agitation and boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 min.

3

Check the pH of each preparation after it is sterilized, which should be between 7.2 and 7.4 at room temperature. This is done by macerating a small amount of medium in a little distilled water or by allowing a little amount of medium to gel around a pH meter electrode.

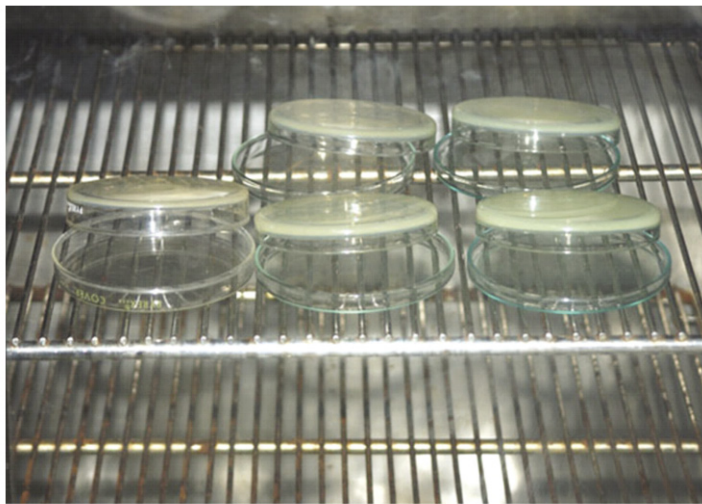


4

Cool the agar medium to 40-50°C. Pour the agar into sterile glass or plastic petri dish on a flat surface to a uniform depth of 4 mm.

5

Allow to solidify.



6

Prior to use, dry plates at 30-37°C in an incubator, with lids partly ajar, for not more than 30 minutes or until excess surface moisture has evaporated. Media must be moist but free of water droplets on the surface. Presence of water droplets may result to swarming bacterial growth, which could give inaccurate results. They are also easily contaminated.

Storage

1

If plates are not to be immediately used, they may be stored in the refrigerator inside airtight plastic bags at 2-8°C for up to 4 weeks.



2

Unpoured media may be stored in airtight screw-capped bottles under the conditions specified by the manufacturer.

Control

Before use, check the ability of the agar to support the growth of control strains (listed in the Introduction) by streaking bacterial cultures on the agar medium. It is also advisable to check the ability of each batch of media to support the growth of a representative member of the species to be tested.

INOCULUM

Preparation

1

From a pure bacterial culture (not more than 48 hours, old except for slow growing organisms), take four or five colonies with a wire loop.



2

Transfer colonies to 5 ml of Trypticase soy broth or 0.9% saline.



3

Incubate the broth at 30°C or at an optimum growth temperature until it achieves or exceeds the turbidity of 0.5 MacFarland standard (prepared by adding 0.5 ml of 0.048 M BaCl₂ to 99.5 ml of 0.36 NH₂SO₄; commercially available).



4

Compare the turbidity of the test bacterial suspension with that of 0.5 MacFarland (vigorously shaken before use) against a white background with contrasting black line under adequate light. Arrow points to tube with correct turbidity.

5

Reduce turbidity by adding sterile saline or broth.

NOTE: Standardized inoculum has a concentration of $1-2 \times 10^8$ cfu/ml.

Inoculation of plates

1

Dip a sterile cotton swab into the standardized bacterial suspension.

**2**

Remove excess inoculum by lightly pressing the swab against the tube wall at a level above that of the liquid.

3

Inoculate the agar by streaking with the swab containing the inoculum.



4

Rotate the plate by 60° and repeat the rubbing procedure. Repeat two times. This will ensure an even distribution of the inoculum.

5

Allow the surface of the medium to dry for 3-5 minutes but not longer than 15 minutes to allow for absorption of excess moisture.

ANTIMICROBIAL DISKS

Selection

The number of antimicrobial agents to be tested should be limited. To make the test practical and relevant, include only one representative of each group of related drugs; those indicated for veterinary use to control or prevent disease, and those that can be useful for epidemiological or research purposes.

Use antibiotic disks purchased from a reputable manufacturer. The disk diameter is approximately 6 mm. Disks should be properly stored in a tightly sealed container with desiccant at 2-8°C. Expired disks should not be used.

Application



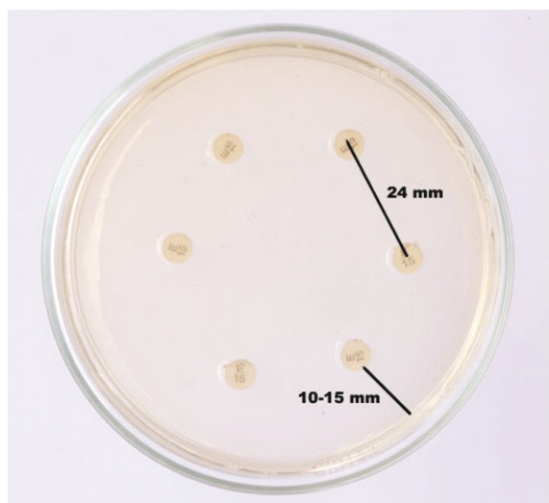
1

Using sterile forceps or disk dispenser, place antibiotic disk on the surface of the inoculated and dried plate.



2

Immediately press it down lightly with the instrument to ensure complete contact between the disk and the agar surface. Do not move a disk once it has come into contact with the agar surface since some diffusion of the drug occurs instantaneously.



3

Position disks such that the minimum center - center distance is 24 mm and no closer than 10 to 15 mm from the edge of the petri dish. A maximum of six disks may be placed in a 9-cm petri dish and 12 disks on a 150 mm plate. Reduce the number of disks applied per plate if overlapping zones of inhibition are encountered.

CONTROL PLATE

Include one plate inoculated with a control strain (Appendix 2.1) for every set of plates and incubate together.

INCUBATION

1

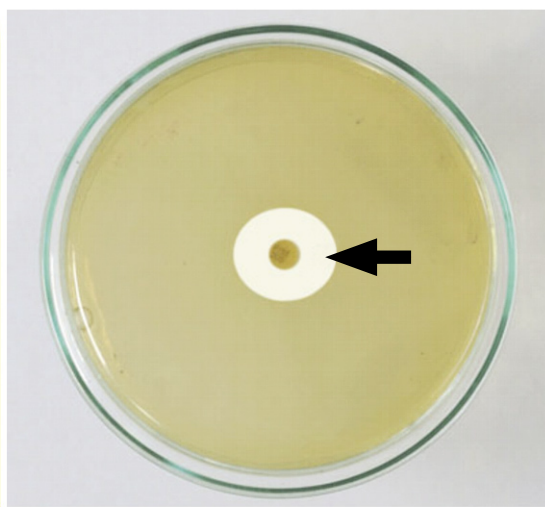
Incubate plates in an inverted position at 30°C or at an optimum growth temperature.

2

Observe for the zone of inhibition after 16 to 18 hours. Slow growing organisms may require longer incubation period.

READING AND MEASUREMENT OF ZONES OF INHIBITION

Description

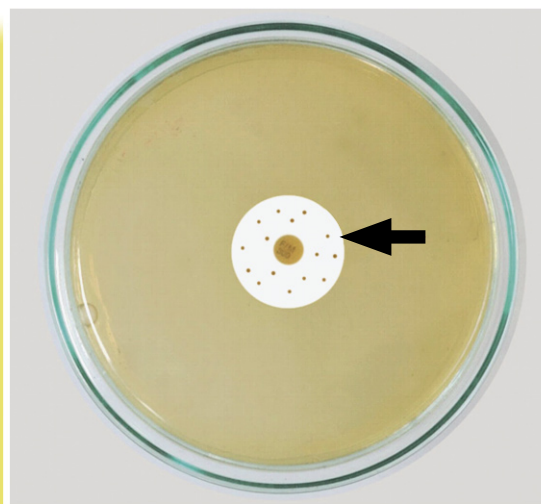


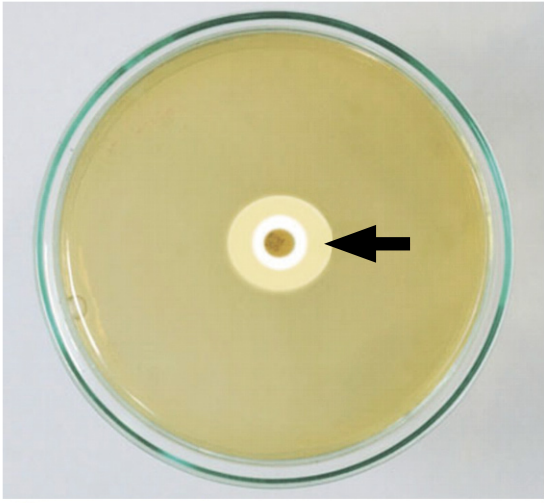
1

The zone of inhibition (arrow) is the point at which no growth is visible to the unaided eye.

2

Record the presence of individual colonies (arrow) within zones of inhibition.





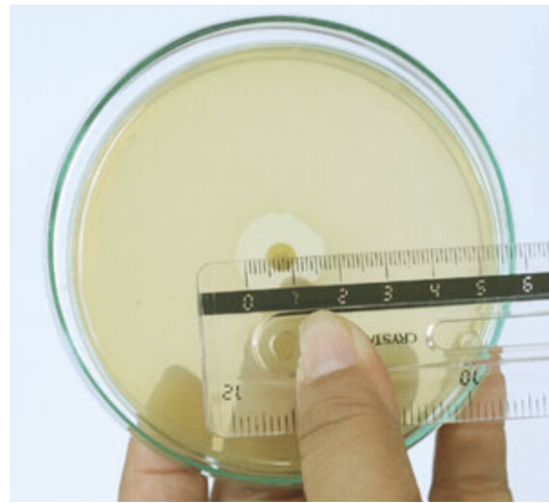
3

Record occurrence of fuzzy zones (arrow). In measuring the zone diameter, the fuzzy portion of the zone should be ignored as much as possible. The zone limit is the inner limit of the zone of normal growth.

Reading

1

Read and record the diameter of the zones of inhibition using a ruler graduated to 0.5 mm.



2

Round up the zone measurement to the nearest millimeter.

INTERPRETATION OF RESULTS

1 Compare the diameter of the zone of inhibition of the test isolates with those in the chart of interpretative standard for veterinary pathogens (Appendix 2.2).

2 Report result as Resistant (R), Intermediate (I) or Susceptible (S).

Example

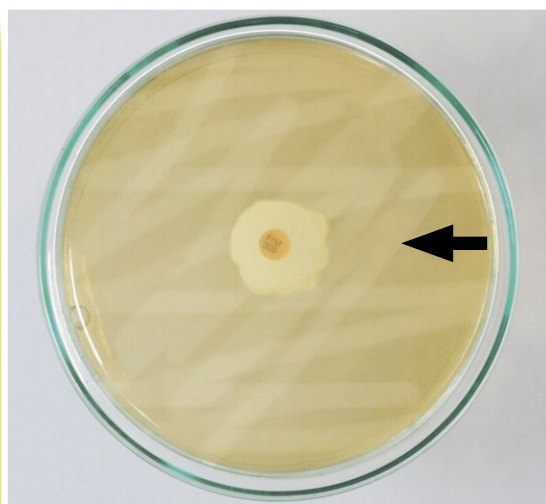
Disk used: Chloramphenicol, 30 μ g (C-30)

Zone of inhibition: 16 mm

Result/ interpretation: Intermediate \rightarrow based on the zone diameter interpretative chart (Appendix 2.2)

3 Susceptibility test results using agents other than those listed in the chart are interpreted on the basis of the presence or absence of a definite zone of inhibition and is considered only as qualitative until such time as interpretative zones have been established.

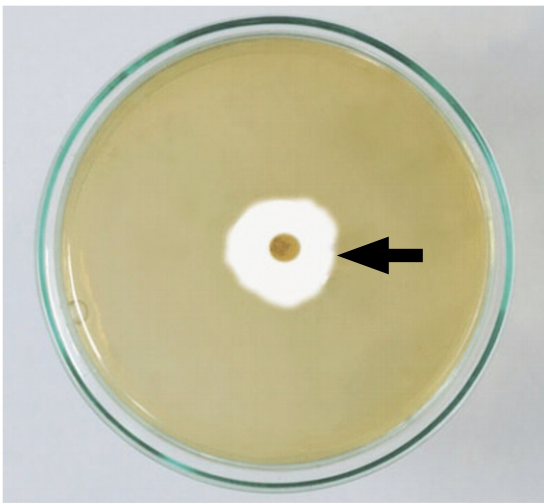
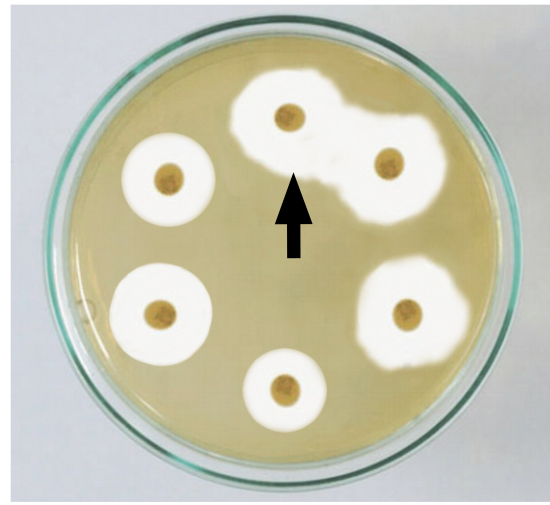
REJECTION CRITERIA



1 Do not read plates on which growth of test bacteria have isolated colonies or less than semi-confluent growth (arrow).

2

Do not read zones of inhibition of two adjacent disks that overlap (arrow) to the extent that measurement of the zone diameter cannot be made.



3

Do not read zones showing distortion from circular (arrow).

4

Reject all data collected in a particular set if the zones of inhibition produced on plate inoculated with a control strain are not within the tolerance limits set.

REFERENCES

- Alderman, D.J. and P. Smith. 2001. Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture*, 196: 211- 243.
- Anonymous. 1986. *Antimicrobial Susceptibility Testing: A System for Standardisation*. Becton Dickinson and Company, Hong Kong, 13 pp.
- Bailey, W.R. and E.G. Scott. 1966. *Diagnostic Microbiology*, Second Edition. Toppan Company Ltd., Japan, pp. 257- 270.
- Finegold, S.M. and W.J. Martin. 1982. *Bailey and Scott's Diagnostic Microbiology*, Sixth Edition. The CV Mosby Company, London, pp. 532- 557.
- NCCLS. 2002. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-Second Edition*. NCCLS document M31-A2 (ISBN 1-56238-461-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA
- Prescott, L.M., J.P. Harley and D.A. Klein. 1993. *Microbiology*, Second Edition. Wm C Brown Publishers, England, pp. 325-343.

APPENDIX 2.1. Acceptable inhibitory zone diameter (mm) limit of control strains recommended for use in the disk diffusion test of antimicrobial sensitivity testing of bacteria isolated from animals.

Antimicrobial	Disk	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Streptococcus pneumoniae</i> ^a
Agent	Content	ATCC 25922	ATCC 25923	ATCC 27853	ATCC 49619
Amikacin	30 µg	19-26	20-26	18-26	-
Amoxicillin-Clavulanic acid ^b	20/10µg	18-24	28-36	-	-
Ampicillin	10µg	16-22	27-35	-	30-36
Cefazolin	30µg	21-27	29-35	-	-
Cefoxitin	30µg	23-29	23-2	-	-
Cephalothin	30 µg	15-21	29-37	-	26-32
Chloramphenicol 30µg	21-27	19-26	-	26-32	-
Clindamycin	2 µg	-	24-3	-	19-25
Erythromycin	15µg	-	22-30	-	25-30
Gentamicin	10µg	19-26	19-27	16-21	-
Imipenem	10µg	26-32	-	20-28	-
Kanamycin	30µg	17-25	19-26	-	-
Oxacillin	1µg	-	18-24	-	≤12 ^c
Penicillin	10 units	-	26-37	-	24-30
Rifampin	5µg	8-10	26-34	-	25-30
Tetracycline	30µg	18-25	24-30	-	27-31
Ticarcillin	75µg	24-30	-	21-27	-
Ticarcillin-Clavulanic acid	75/10µg	24-30	29-37	20-28	-
Spectinomycin	100 µg	21-25	13-17	10-14	-
Sulfisoxazole	250 µg or 300 µg	15-23	24-34	-	-
Trimethoprim-Sulfamethoxazole ^d	1.25/23.75 µg	23-29	24-32	-	20-28
Vancomycin	30µg-	-	17-21	-	20-27

* Adapted from M31-A2 NCCLS. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-Second Edition. NCCLS document M31-A2 (ISBN 1-56238-461-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

- no established range.

a applicable only using Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood, incubated in 5% CO₂.

b range for E. coli ATCC 35218 is 17-22 mm.

c best assessed using Staphylococcus aureus ATCC 25923 with acceptable zone diameter of 18-24 mm.

d very medium-dependent specially with enterococci.

NOTE: Reproduced with permission; from NCCLS publication M31-A2-Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals: Approved Standard-Second Edition (ISBN 1-56238-461-9). Copies of the current edition may be obtained from NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

APPENDIX 2.2. Zone diameter interpretative standard for veterinary pathogens.

Antimicrobial Agent	Disk Content	Zone Diameter (mm)			
		S	I	F	R
Amikacin*	30 μ g	≥ 17	15-16		≤ 14
Gentamicin*	10 μ g	≥ 15	13-14		≤ 12
Kanamycin*	30 μ g	≥ 18	14-17		≤ 13
Spectinomycin	100 μ g	≥ 14	11-13		≤ 10
Amoxicillin-clavulanic acid*					
Staphylococci	20/10 μ g	≥ 20	-		≤ 19
Other organisms	20/10 μ g	≥ 18	14-17		≤ 13
Ticarcillin-clavulanic acid*					
<i>Pseudomonas aeruginosa</i>	75/10 μ g	≥ 15	-		≤ 14
Gram(-)enteric organisms	75/10 μ g	≥ 20	15-19		≤ 14
Ampicillin*					
Enterobacteriaceae	10 μ g	≥ 17	14-16		≤ 13
Staphylococci	10 μ g	≥ 29	-		≤ 28
Enterococci	10 μ g	≥ 17	-		≤ 16
Streptococci (not <i>S. pneumoniae</i>)	10 μ g	≥ 26	19-25		≤ 18
Oxacillin*					
Staphylococci	1 μ g	≥ 13	11-12		≤ 10
Penicillin*					
Staphylococci	10 units	≥ 29	-		≤ 28
Enterococci	10 units	≥ 15	-		≤ 14
<i>S. pneumoniae</i>	1 μ g oxacillin	≥ 20	-		-
Streptococci (not <i>S. pneumoniae</i>)	10 units	≥ 28	20-27		≤ 19
Ticarcillin*					
<i>Pseudomonas aeruginosa</i>	75 μ g	≥ 15	-		≤ 14
Gram (-) enteric organisms	75 μ g	≥ 20	15-19		≤ 14
Penicillin-novobiocin	10 units/30 μ g	≥ 18	15-17		≤ 14
Imipenem*	10 μ g	≥ 16	14-15		≤ 13
Cephalothin*	30 μ g	≥ 18	15-17		≤ 14
Cefazolin*	30 μ g	≥ 18	15-17		≤ 14
Ceftiofur	30 μ g	≥ 21	18-20		≤ 17
Enrofloxacin (canine/feline)	5 μ g	≥ 23	-	17-22	≤ 16
Enrofloxacin (chickens/turkeys)	5 μ g	≥ 23	17-22		≤ 16
Enrofloxacin (bovine)	5 μ g	≥ 21	17- 20		≤ 16
Difloxacin	10 μ g	≥ 21	18- 20		≤ 17
Orbifloxacin	10 μ g	≥ 28	-	18-22	≤ 17

APPENDIX 2.2. Continuation

Antimicrobial Agent	Disk Content	Zone Diameter (mm)			
		S	I	F	R
Clindamycin	2µg	≥ 21	15-20		≤ 14
Pirlimycin	2µg	≥ 13	-		≤ 12
Erythromycin*					
Streptococci	15µg	≥ 21	16-20		≤ 15
Organisms other than Streptococci	15µg	≥ 23	14-22		≤ 13
Tilmicosin (Bovine)	15µg	≥ 14	11-13		≤ 10
Tilmicosin (Swine)	15µg	≥ 11			≤ 10
Chloramphenicol*					
Streptococci (not <i>S. pneumoniae</i>)	30µg	≥ 21	18-20		≤ 17
<i>S. pneumoniae</i>	30µg	≥ 21	-		≤ 20
Organisms other than Streptococci	30µg	≥ 18	13-17		≤ 12
Florfenicol	30µg	≥ 19	15- 18		≤ 14
Tiamulin	30µg	≥ 9	-		≤ 8
Trimethoprim-sulfamethoxazole*					
<i>Streptococcus pneumoniae</i>	1.25/23.75µg	≥ 19	16-18		≤ 15
Organisms other than <i>S. pneumoniae</i>	1.25/23.75µg	≥ 16	11-15		≤ 10
Rifampin*					
<i>Streptococcus pneumoniae</i>	5	≥ 19	17-18		≤ 16
Organisms other than Streptococci	5	≥ 20	17-19		≤ 16
Sulfisoxazole*	250 or 300	≥ 17	13- 16		≤ 12
Tetracycline*					
Streptococci	30	≥ 23	19-22		≤ 18
Organisms other than Streptococci	30	≥ 19	15-18		≤ 14
Vancomycin*					
Enterococci	30	≥ 17	15-16		≤ 14
Streptococci	30	≥ 17	-		-
Other gram-positive organisms	30	≥ 12	10-11		≤ 9

* human data taken from M100-S12 supplements to M2 and M7

S Susceptible

I Intermediate

R Resistant

F Flexible; should be considered susceptible if appropriate dosing modifications specified in the packaging insert are applied

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CHAPTER 3

Minimal Inhibitory Concentration (MIC) Test and Determination of Antimicrobial Resistant Bacterial

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PRINCIPLE

The agar dilution technique is used to measure qualitatively the *in vitro* activity of an antimicrobial agent against the test bacteria. In this method, graded amounts of antibiotics are incorporated in agar plates and inoculated in spots with the organisms under study. If the organism under study is susceptible to the incorporated antibiotic, no bacterial growth is expected in agar plates with higher amounts of the drugs. Bacterial growth is observed as the antibiotic concentration in the agar plate diminishes. Inhibition of growth at the minimum or lowest concentration of antibiotic is regarded as the end point.

MEDIA

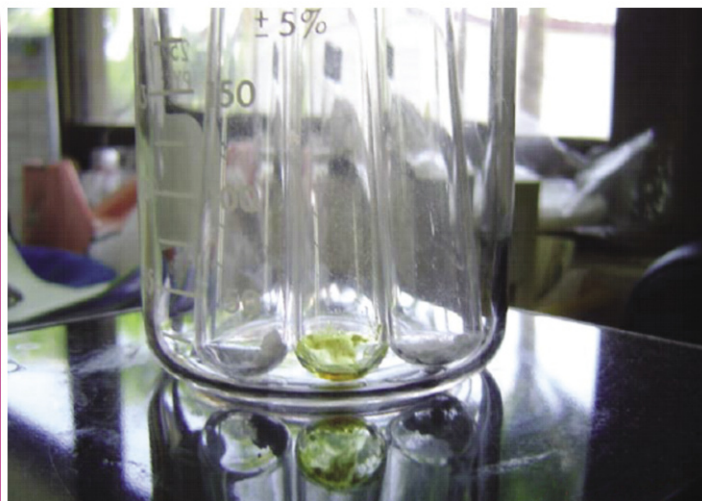
Refer to Appendix 3.1 for the different culture media, solvents, antimicrobial agents, control strains and apparatus needed for the minimal inhibitory concentration (MIC) test.

The minimal inhibitory concentration (MIC) test is performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility, low in sulfonamide, trimethoprim, and tetracycline inhibitors, and gives satisfactory growth of most bacterial pathogens.

The inoculum is prepared using a suitable broth such as heart infusion broth (HIB). This medium is prepared according to the manufacturer's instructions, dispensed in tubes at 3 ml and sterilized. Sterile 0.9% salt solution may also be used.

Media are supplemented with 1-2% sodium chloride (NaCl) if intended for marine species.

Preparation of antimicrobial agent stock solution

**1**

Remove the antimicrobial agent from the freezer, and warm to room temperature before opening to avoid condensation of water.

2

Weigh appropriate amount of the powdered antimicrobial agent.

NOTE: *The standard units of activity of the antimicrobial agents may differ widely by the actual weight of the powder or may differ within a drug production lot. Each laboratory must standardize the antimicrobial agent solutions whenever they are being used. See Appendix 3.2 for the formula used to determine the amount of powder needed for a standard solution.*

3

Dissolve the antimicrobial agent powder in solvent to make a concentration of 1,000 $\mu\text{g/ml}$.

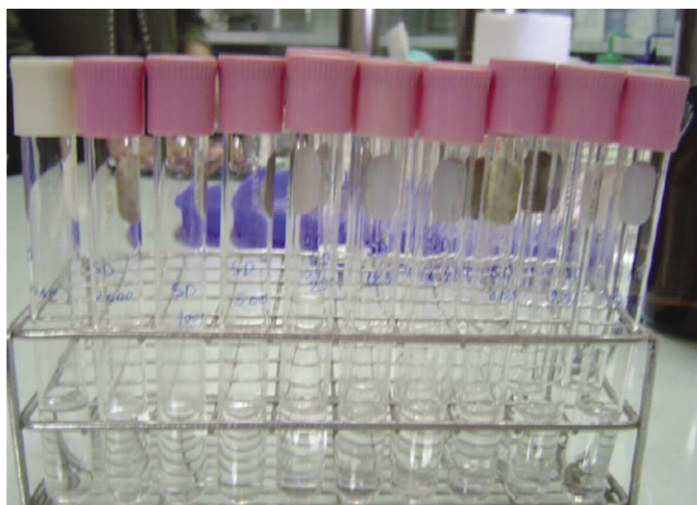


NOTE: *Stock Solutions*

- a. *At least 1,000 $\mu\text{g/ml}$ or 10 times the highest concentration to be tested is to be prepared as an antimicrobial agent stock solution. Some antimicrobial agents are of limited solubility. Therefore, lower concentration may be required. However, in all cases, drug manufacturers provide directions for determining solubility.*
- b. *Some drugs must be dissolved in solvents other than water. In such cases, it is necessary to:*
 - 1) *Use only enough solvent to dissolve the antimicrobial agent powder;*
 - 2) *Dilute to the final stock concentration with distilled water as indicated in Appendix 3.3;*
 - 3) *Prepare appropriate volumes of distilled water, the desired volume for serial dilution.*

- 4** Dispense the stock solution into sterile diluent using two-fold dilution technique.

NOTE: As an example, a range of 0.012-100 µg/ml two-fold dilutions may be used as test concentrations for different antibiotics. For Sulfadimethoxine and Sulfadiazine, 400 µg/ml may be used as the highest test concentration (see Appendix 3.4).

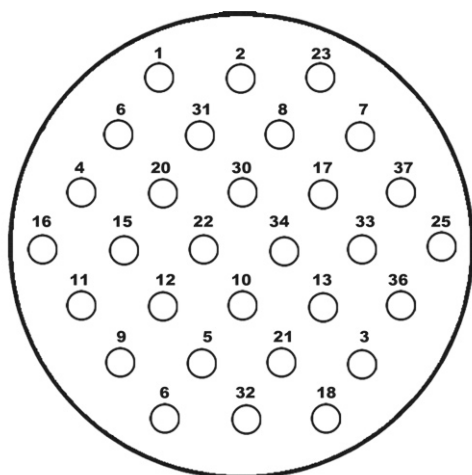


- 5** Set aside.

Preparation of antimicrobial agar plates

- 1** Label each empty sterile plate in order to identify the antimicrobial agent and their concentrations.

Example: 0.024 OTC means 0.024 µg/ml of oxytetracycline
- 2** Place the label on the upper portion of the bottom side of the petri dish to ensure that the plate is inserted at the correct point of the basal stand A of the multiple inoculating apparatus.



3

Draw a scheme to locate each bacterial strain in a well on a reference paper that will be used to read the results.

4

Prepare MHA following manufacturer's direction and supplement with 1-2% NaCl, if intended for marine organisms. Keep in a water bath at 48-50°C until use.

5

Pipette 1 ml of appropriate dilutions of the test antimicrobial agent (previously prepared, page 32) into the labeled plate (prepared in number 1). Two replicates must be made for each concentration.





- 6** Pipette 9 ml of MHA (keep warm at 48-50°C), add into the plate with appropriate dilution of the test antimicrobial agent and mix thoroughly.

- 7** Allow the agar to solidify at room temperature and use the plates immediately after the agar surface has dried completely. If necessary, dry the surface of agar in a laminar flow chamber under UV light, but avoid excessive drying.



Control agar plates/Drug-free agar plates

Prepare control agar plates by pipetting 10 ml of MHA into a sterile petri dish. Do not add any antimicrobial agent. There should at least be 2 control plates.

INOCULUM

Preparation

- 1 From a pure 18-24 hour bacterial culture get 4-5 isolated colonies (to minimize the risk of picking bacteria which have lost their resistance) and subculture to a tube with 3 ml HIB containing 0.3% yeast extract and 2% NaCl.



2

Shake vigorously in a water bath at 30°C until it achieves or exceeds the turbidity of 0.5 MacFarland standard (prepared by adding 0.5 ml of 0.048 M BaCl_2 to 99.5 ml of 0.36 NH_2SO_4 ; commercially available). The inoculum may also be standardized based on optical density [OD_{625} of 0.08-0.1 (1cm light path)] using a spectrophotometer. This is usually achieved after 18-24 hours.



NOTE: Standardized inoculum has a concentration of $1-2 \times 10^8$ cfu/ml.



3

Dilute the standardized inoculum 1:10 in sterile saline solution to obtain the desired concentration of 10^6 cfu/ml.

4

Pipette 0.1 ml of the 10^6 cfu/ml inoculum and transfer to a well, of a multi-dispenser containing 0.9 ml of HIB with 0.3% yeast extract.

In the absence of a multi-dispenser (photo at right), sterile test tubes of the same size, may be used to hold the diluted standardized inoculum.



NOTE: The final concentration of the bacterial suspension is approximately 10^5 cfu/ml well.

5

Store at room temperature for not more than 2 hours before testing.

Inoculation volume

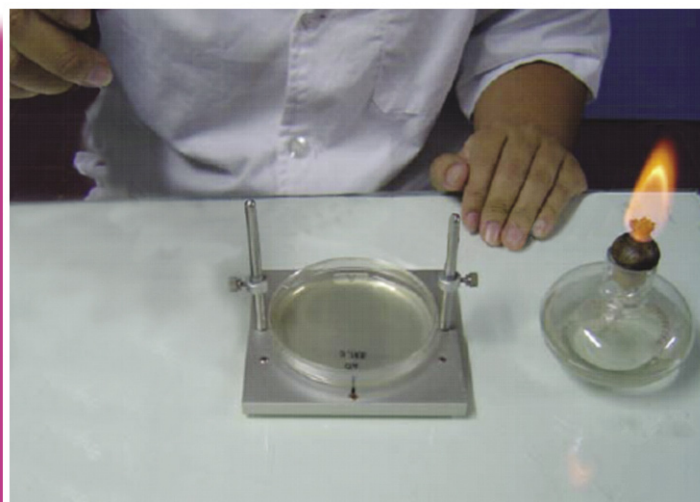
Inoculate plates with 1-3 μl of the inoculum if automatic multi-dispenser is used and 10 μl if done manually. Inoculated volume should be uniform for good results.

Inoculation sequence

- 1** When using automatic multiple inoculating apparatus, drug free or control plate should be inoculated first, followed by the plate with the lowest antimicrobial concentration. Inoculate a second control agar plate last to ensure that there is no contamination or antimicrobial agent carry-over during the inoculation.
- 2** When inoculating manually, it is only important to include a drug free or control plate at the beginning of the inoculation series.

Inoculation

- 1** Place a completely dried agar plate on the basal stand A of the multiple inoculating apparatus. Insert the plate on the basal stand A of the multiple inoculating apparatus in such a way that the label on the plate is facing the front direction.

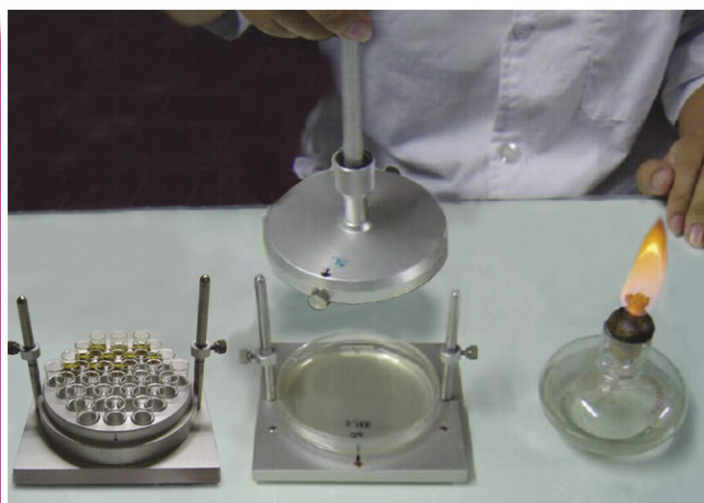


**2**

Place the multiple well tray of the multi-dispenser, which contain 1 ml of inocula, onto the basal stand B to ensure that the marking on the tray is arranged at the front direction.

3

Set the tray to ensure that each inoculator stick will properly dip into the inoculum and carry a uniform volume of each test bacteria.

**4**

Set the apparatus to ensure that the surface of each multiple inoculator stick will properly touch the surface of the medium in agar plate.

5

Inoculate the bacterial suspensions onto the surface of the agar plate.



CONTROL PLATE

Include a control strain on each plate (see Appendix 3.1).

INCUBATION

1

Let the inoculated agar plates remain at room temperature until the moisture in the inoculum spot is absorbed by the agar or until all spots are dry.

2

Incubate the plates in an inverted position at 30°C for 18-24 hours

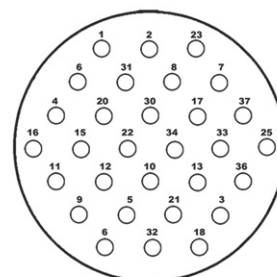


NOTE: The incubation time is extremely important to obtain reliable end points when reading the results.

READING OF MIC VALUES

1

Lay the agar plates on a non-reflecting dark surface and observe bacterial growth with the naked eye. Place the inoculated plate on or beside the reference paper to identify the spot or position of each tested bacteria.



2

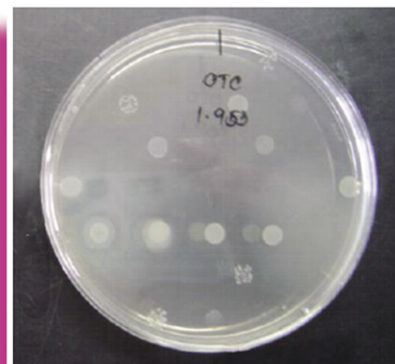
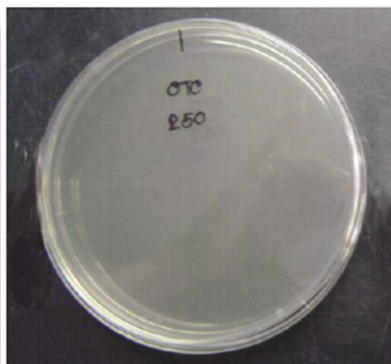
Check bacterial growth on the 2 control plates, disregard the results if no growth is observed in some control strains. The test should be repeated.

3

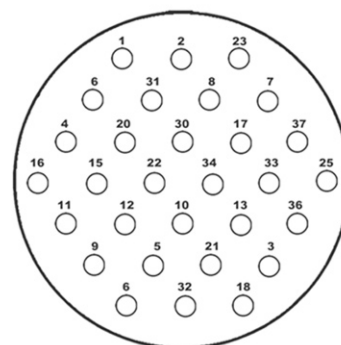
Read and record the MIC at the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the naked eye.

Example:

a) No growth in plates with 250 $\mu\text{g/ml}$ OTC, this means that OTC at 250 $\mu\text{g/ml}$ completely inhibited all strains.



b) Based on the reference paper, spots inoculated with bacterial strains # 1, 2, 31, 4, 30, 37, 15, 22, 34, 33, 36, 5, 31, 3 and 18 did not grow in plates with 1.953 $\mu\text{g/ml}$ OTC, this means that OTC at 1.953 $\mu\text{g/ml}$ inhibited the growth of these strains.



- 4 The MIC is the lowest concentration of antimicrobial agent that completely inhibits colony formation.
- 5 Disregard a single colony or a faint haze caused by the inoculum.
- 6 Repeat the test if two or more colonies persist in concentrations of the agent beyond an obvious end point; that is, if there is no growth at lower concentration but there is growth at higher concentrations.
- 7 When reporting the MIC values of antimicrobial agents that will prevent the growth of bacterial strains, include important information such as the locality and date the samples were isolated or collected.
- 8 Evaluate the MIC range of each antimicrobial agent.
- 9 Compare the MIC breakpoint of the test isolates with those in the chart (Appendix 3.5).
- 10 Report result as Resistant (R), Intermediate (I) or Susceptible (S).

Example:

Antibiotic:	oxytetracycline
MIC breakpoint:	0.2 µg/ml
Interpretation:	susceptible

NOTE: The “true” MIC value is the lowest test concentration that completely inhibits the growth of organisms in both replicate plates. However, if the “observed” MIC values of the tested plates are different, for example, two-fold dilutions were used to test OTC and the “observed” MIC value of OTC in the first plate was 6.25 µg/ml, and the second plate was 3.125 µg/ml, the “true” MIC of OTC would be between 6.25 µg/ml and 3.125 µg/ml.

Determination of Antimicrobial Resistant Bacteria

This activity will determine and provide baseline data on bacterial drug resistance that could be useful to policy makers and surveillance teams or systems.

INHIBITORY CONCENTRATION AT 50% AND 90%

After the MIC values are read, further interpretation of the inhibitory concentration at 50% (IC_{50}) and 90% (IC_{90}) of the bacterial strains are carried out.

Basically, the IC_{50} or IC_{90} is used to determine the dosage regimen of antimicrobial agent for *in vivo* treatment against the pathogen in the field to know the effectiveness of each drug. The formula used to determine IC_{50} and IC_{90} values are presented in Appendix 3.6.

When the IC_{50} and IC_{90} values could not be determined from any value of the MIC series results, these must be determined from the average value between the nearest percent that is lower and higher than the IC_{50} and IC_{90} values (see Appendix 3.6).

DETERMINATION OF ANTIMICROBIAL RESISTANT BACTERIA

The results for antimicrobial resistant bacteria can be evaluated according to the NCCLS standard for interpretative categories and correlative minimal inhibitory concentration (MICs) (NCCLS, 1998). Since the NCCLS standard does not include breakpoint recommendations for all of the agents tested, and the data are based on human drugs, which are recently used in shrimp production activity, therefore, data shown in Appendix 3.5 are standard categories used for determining drug resistance for bacteria which are close to food borne pathogenic bacteria, e.g., *Vibrio cholerae*.

In case a number of bacterial strains have been tested using MIC standardized method, the MIC values obtained from the study can be used to determine the breakpoint of each agent and interpretation of results for drug resistance.

The following steps should be applied:

- 1** A large number of bacterial strains (at least 300 strains) should be tested for each antimicrobial agent using MIC standardized method.
- 2** Arrange the MIC values of each bacterial strain against an agent and fill into the assigned table (see example in Appendix 3.7).

3

Analyze the results by using a program in WHONET to evaluate the breakpoint of each antimicrobial agent. To access the program: go to www.who.int, click drug resistance on the right side of the web page. On the left side of the web page is the table of contents, click # 2 How to obtain WHONET 5 through the internet. This will give you instructions on how to download the software.

4

Determine the resistant bacterial strains according to the breakpoint of each antimicrobial agent.

Some notes on laboratory practices are presented in Appendix 3.8.

REFERENCES

- Alderman, D.J. and P. Smith. 2001. Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture*, 196: 211- 243.
- Baker, C.N., C. Thornsberry and R.W. Hawkinson. 1983. Inoculum standardization in antimicrobial susceptibility tests: evaluation of the use of overnight agar cultures and the rapid inoculum standardization system. *Clin. Microbiol.*, 17: 450-457.
- Ericsson, H.M. and J.C. Sherris. 1971. Antibiotic sensitivity testing. Report of an international study. *Acta Pathol. Microbiol. Scand. B*, 217 (suppl.): 1-90.
- Japan Society of Chemotherapy. 1981. Method of minimal inhibitory concentration. *Chemotherapy*, 29: 76-78.
- Jones, R.N., A.L. Barry, T.L. Gavan and J.A. Washington. 1985. Susceptibility tests: Microdilution and macrodilution. In: Lennette, E.M., Balows, A., Hausler, W.J. and Shadomy, H.J. (eds.), *Manual of Clinical Microbiology*. Fourth Edition. American Society for Microbiology, Washington, DC, USA, pp. 972-977.
- McDonald, C.L., W.E. Maher and R.J. Fass. 1995. Revised interpretation of oxacillin MICs for *Staphylococcus epidermidis* based on MecA detection. *Antimicrob. Agents Chemother.*, 39: 982-984.
- NCCLS. 1998. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests of Bacteria Isolated from Animals; Approved Standard. NCCLS Document M31-A, NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087, USA.

- NCCLS. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard, Second Edition. NCCLS document M31-A2 (ISBN 1-56238-461-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.
- Thornsberry, C., T.L. Gaven and E.H. Gerlach. 1977. New developments in antimicrobial agent susceptibility testing. *Cumitech*, 6: 1-13.
- Thornsberry, C. and L.K. McDougal. 1983. Successful use of broth microdilution in susceptibility tests for methicillin-resistant (heteroresistant) staphylococci. *Clin. Microbiol.*, 18: 1084-1091.
- Watts, J.L. and S.C. Nickerson. 1986. Evaluation of a rapid inoculum standardization system of antibiotic susceptibility testing of bovine mammary gland isolates. *Vet. Microbiol.*, 2: 269-276.

APPENDIX 3.1. List of culture apparatus, culture media, solvents, control strains, and antimicrobial agents needed for the minimal inhibitory concentration (MIC) test.

Apparatus:

1. MIC multiple inoculating apparatus set
2. Autoclave
3. Incubator
4. Analytical balance
5. Laminar flow hood
6. Water bath shaker
7. Refrigerator
8. Micropipette (20-100 and 200-1000 μ l)
9. Vortex mixer
10. Glass pipette 2, 5, 20 ml
11. Test tubes
12. Dissecting set
13. Petri dishes (glass and disposable)

Culture media:

1. Thiosulphate-citrate-bilesalt-sucrose agar (TCBS, Difco)
2. Glutamate starch phenol-red agar (GSP, Merck)
3. Muller Hinton Agar (MHA, Merck)
4. Tryptic soy agar (TSA, Difco)
5. Sterile normal saline solution (NSS)
6. Heart infusion agar (HIA, Difco)
7. Heart infusion broth (HIB, Difco)
8. Sodium chloride (NaCl)

Solvents:

1. Distilled water
2. 0.1 N sodium hydroxide (NaOH)
3. Absolute ethanol
4. Dimethyl sulfoxide
5. N-N-Dimethyl formamide

APPENDIX 3.1. Continuation**Control bacterial strains:**

1. *Enterococcus faecalis* ATCC 29212
2. *Escherichia coli* ATCC 25922

Antimicrobial agents:

1. Chloramphenicol (CP)
2. Erythromycin (E)
3. Furazolidone (FD)
4. Oxolinic acid (OA)
5. Oxytetracycline (OTC)
6. Norfloxacin (NFX)
7. Prefloxacin (PFX)
8. Trimethoprim (TM)
9. Sulfadiazine (SD)
10. Sulfadimethoxine (S)

NOTE: Each laboratory should consider which agents to routinely test or report, in accordance with the needs of each institute's study plan or of the farmers in particular aqua culture areas. The test reports should help to minimize the resistant strains due to misuse or overuse of the agents. At present, except for CP and FD, the listed antibiotics are FDA approved agents for use in food animal production (NCCLS, 1998).

APPENDIX 3.2. Preparation of antimicrobial agent stock solution.

Formula to determine amount of antimicrobial agent powder needed for a standard solution:

$$\text{Volume (ml)} = \frac{\text{weight (mg)} \times \text{assay potency (}\mu\text{g/mg)}}{\text{concentration (}\mu\text{g/ml)}}$$

Example:

To prepare 10 ml (volume) of a stock solution containing 1,000 $\mu\text{g/ml}$ (desired concentration) of antimicrobial agent with a potency of 94.5 $\mu\text{g/ml}$:

$$\text{Volume} = \frac{\text{actual weight} \times \text{potency}}{\text{desired concentration}}$$

$$10 \text{ ml} = \frac{\text{actual weight (mg)} \times 94.5 \mu\text{g/ml}}{1,000 \mu\text{g/ml}}$$

$$\begin{aligned} \text{Actual weight} &= \frac{1,000}{94.5} \\ &= 105.8201 \text{ mg} \end{aligned}$$

Therefore, 105.8201 mg of powdered antimicrobial agent is needed for a 10 ml stock.

APPENDIX 3.3. List of solvents and diluents needed for the preparation of stock solutions of antimicrobial agents.

Antimicrobial agent	Solvent	Diluent
Oxolinic acid	0.1 N NaOH	Distilled water
Norfloxacin	0.1 N NaOH	Distilled water
Prefloxacin	0.1 N NaOH	Distilled water
Sulfadiazine	0.1 N NaOH	Distilled water
Sulfadimethoxine	0.1 N NaOH	Distilled water
Chloramphenicol	95 % ethanol	Distilled water
Erythromycin	95 % ethanol	Distilled water
Furazolidone	N-N-Dimethyl formamide	Distilled water
Oxytetracycline	Distilled water	Distilled water
Trimethoprim	Dimethyl sulfoxide or 0.05 N (0.05 mol/L) lactic or hydrochloric acid 10% of final volume	Distilled water

NOTE: For antimicrobial agents which must be dissolved in solvents other than water, it is necessary to follow this guide:

- Use only enough solvent to dissolve the powdered antimicrobial agent (in our case, 1 ml of solvent was used).
- Dilute to the final stock concentration with distilled water.
- If sterile solutions are desired, they are to be filtered through a membrane filter.
- Use antimicrobial solution immediately after preparing and discard unused portion at the end of the day.
- Stock solution of antimicrobial agents can be stored at -70°C or colder for 6 months without significant loss of activity.

Appendix 3.4. Preparation of dilutions of antimicrobial agents for use in the agar dilution method of minimal inhibitory concentration (MIC) test.

Step	Concentration (µg/ml)	Source	Volume (ml)	Distilledwater	Intermediate Conc. (µg/ml)	Final Conc. At 1: 10 dilution in Agar (µg/ml)
1	1000	Stock	10	10	1000	100
2	1000	Step 1	5	5	500	50
3	500	Step 2	5	5	250	25
4	250	Step 3	5	5	125	12.5
5	125	Step 4	5	5	62.50	6.25
6	62.5	Step 5	5	5	31.25	3.125
7	31.25	Step 6	5	5	15.625	1.56
8	15.625	Step 7	5	5	7.812	0.781
9	7.82	Step 8	5	5	3.906	0.390
10	3.906	Step 9	5	5	1.953	0.195
11	1.953	Step 10	5	5	0.977	0.098
12	0.977	Step 11	5	5	0.488	0.0481
13	0.488	Step 12	5	5	0.244	0.024
14	0.244	Step13	5	5	0.122	0.012

NOTE:

- a. *Modified from Ericsson and Sherris (1971).*
- b. *For S and SD, stock solutions were prepared at 4,000 µg/ml and the concentrations were made by the same method as shown in the scheme.*

APPENDIX 3.5. Interpretative categories and correlative minimal inhibitory concentration (MICs) for food-borne pathogens (source: NCCLS, 1998).

Antimicrobial agent	MIC Breakpoint µg/ml		
	Susceptible	Intermediate	Resistant
Chloramphenicol*	≤ 8	-	≥ 32
Oxytetracycline*	≤ 4	-	≥ 16
Erythromycin*	≤ 0.5	-	≥ 8
Furazolidone	≤ 0.5	-	≥ 128
Norfloxacin	≤ 0.5	-	≥ 16
Prefloxacin	≤ 0.5	-	≥ 16
Sulfadiazine	≤ 0.5	-	≥ 152
Sulfadimethoxine	≤ 256	-	≥ 350
Trimethoprim	≤ 2	-	≥ 16
Oxolinic acid	≤ 8	-	≥ 32

* based on NCCLS publication M31-A2- Performance Standards for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals: Approved Standard- Second Edition (ISBN 1-56238-461-9).

NOTE: *The data are based on human drugs.*

APPENDIX 3.6. Determination of inhibitory concentrations at 50% and 90% (IC_{50} and IC_{90} values).

Formula:

$$IC_{50} = \frac{A+B}{2} : \text{when } A = \frac{50 \times \text{MIC value of the next \% lower than 50\%}}{\text{the next \% lower than 50\%}}$$

$$B = \frac{50 \times \text{MIC value of the next \% higher than 50\%}}{\text{the next \% higher than 50\%}}$$

$$IC_{90} = \frac{C+D}{2} : \text{when } C = \frac{90 \times \text{MIC value of the next \% lower than 90\%}}{\text{the next \% lower than 90\%}}$$

$$D = \frac{90 \times \text{MIC value of the next \% higher than 90\%}}{\text{the next \% higher than 90\%}}$$

Example: How to find IC_{50} and IC_{90} of OTC from the following MIC values.

MIC ($\mu\text{g/ml}$)	% of Inhibiting Strains
0.0125	5
0.025	20
0.05	30
*0.1	46 *
**0.2	58 **
0.4	63
0.8	70
*1.6	84 *
**3.2	95 **
6.4	100
100	100

The next lower * and higher ** values of IC_{50}
The next % lower * and higher ** than 50%

The next lower * and higher ** values of IC_{90}
The next % lower * and higher ** than 90%

Calculation for IC_{50}

$$A = \frac{50 \times 0.1}{46} = 0.1087$$

$$B = \frac{50 \times 0.2}{58} = 0.1724$$

$$IC_{50} \text{ of OTC} = \frac{0.1087 + 0.1724}{2} \\ = 0.1406 \mu\text{g/ml}$$

Calculation for IC_{90}

$$C = \frac{90 \times 1.6}{84} = 1.7143$$

$$D = \frac{90 \times 3.2}{95} = 3.0316$$

$$IC_{90} \text{ of OTC} = \frac{1.7143 + 3.0316}{2} \\ = 2.3729 \mu\text{g/ml}$$

APPENDIX 3.7. Table of assigned values to evaluate the breakpoint, which correlates to the minimal inhibitory concentration (MIC).

Example: The MIC values of the bacterial strains against OTC, OA, S, SD, CP and TM in various provinces of Thailand in 2002.

No. of Strain	Source of Sample	MIC Values (µg/ml)					
		OTC	OA	S	SD	CP	TM
01	Chanthaburi	0.2	0.2	>400	>400	0.1	25
02	Chanthaburi	0.4	0.1	200	>400	0.4	50
03	Chanthaburi	0.1	0.4	>400	200	0.4	12.5
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
81	Samutsakhon	0.8	0.2	200	200	0.4	6.28
82	Samutsakhon	0.2	0.4	>400	>400	0.2	3.50
83	Samutsakhon	0.2	0.1	>400	200	1.25	25
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
150	Phuket	0.1	0.2	>400	>400	0.2	50
151	Phuket	0.1	0.4	>400	>200	0.4	25
152	Phuket	0.4	0.2	200	>400	0.4	25

APPENDIX 3.8. Notes on laboratory practice (adapted from NCCLS, 2002)**1) Guidelines for Quality Control**

The goal of a quality control program is to monitor the following:

- a. The precision and accuracy of the MIC test procedure;
- b. The performance of reagents and the viability of the microorganisms used in the test; and
- c. The performance of personnel who carry out the tests and interpret the results.

2) Responsibilities of the Laboratory

The following are the responsibilities of the laboratory:

- a. Storage to prevent drug deterioration;
- b. Personnel proficiency;
- c. Adherence to procedure [e.g., inoculum standards, incubation conditions such as time, temperature, and atmosphere (aerobic or anaerobic)]; and
- d. Media preparation and storage.

3) Common Sources of Error

The following are common sources of error and should be checked whenever the results of MIC are outside the accuracy control limits:

- a. Reader error in observation of the apparent bacterial growth on each concentration;
- b. Contamination or other changes in the control plates;
- c. Inoculum adjusted too heavy or too light;
- d. Failure to thoroughly mix the antimicrobial dilution tube;
- e. Variability in the performance of antibiotic media. Each new lot should be checked before use;
- f. Loss of drug potency during handling or storage in the laboratory; and
- g. Mistake in labeling antimicrobial concentration and/or bacterial code.

4) Safety in Laboratory Practice

Carry out all procedures in accordance with the local code of practice of a microbiological laboratory such as ISO/IEC standard.